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Simpson-Golabi-Behmel Syndrome: Genotype/Phenotype Analysis of 18 Affected Males From 7 Unrelated Families

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Simpson-Golabi-Behmel syndrome (SGBS) is an X-linked overgrowth disorder recently shown to be caused by mutations in the heparan sulfate proteoglycan GPC3 [Pilia et al., Nat Genet; 12:241–247 1996]. We have used Southern blot analysis and polymerase chain reaction amplification of intra-exonic sequences to identify four new GPC3 mutations and further characterize three previously reported SGBS mutations. De novo GPC3 mutations were identified in 2 families. In general, the mutations were unique deletions ranging from less than 0.1 kb to more than 300 kb in length with no evidence of a mutational hot spot discerned. The lack of correlation between the phenotype of 18 affected males from these 7 families and the location and size of the GPC3 gene mutations suggest that SGBS is caused by a nonfunctional GPC3 protein.

KEY WORDS: Simpson-Golabi-Behmel syndrome, overgrowth, gene mutation, GPC3, MXR7, heparan sulfate proteoglycan, IGF2, chromosome Xq26

INTRODUCTION

Recently membrane-associated heparan sulfate proteoglycan (GPC3) was implicated as the cause of the X-linked overgrowth disorder called Simpson-Golabi-Behmel syndrome (SGBS) [Pilia et al., 1996]. Proteoglycans are essential cofactors in receptor-growth factor interactions, in cell-cell recognition systems, and in cell-matrix adhesion processes [reviewed by David, 1993]. GPC3 is the human homologue of the developmentally regulated rat intestinal gene OCI-5, with 90% nucleotide and 94% amino acid homology [National Center for Biotechnology Information (NCBI) BLASTX Program (Altschul et al., 1990; Gish and States, 1993)]. The human homologue (also known as MXR7) was first cloned by Lage [1994]. OCI-5 and GPC3 are glypican-related integral membrane proteoglycans (GRIPS) that are anchored to the cell membrane via covalent linkage to glycosyl-phosphatidylinositol (GPI) [David, 1993; Filmus et al., 1995]. Five GRIPS have been cloned: human, mouse, and rat GPC3 [Filmus et al., 1988; Lage, 1994; Watanabe et al., 1995; Pilia et al., 1996]; human, mouse, and rat glypican [Lories et al., 1987; David et al., 1990; Karthikeyan et al., 1992; Watanabe et al., 1995]; cerebroglycan [Stipp et al., 1994]; K-glypican [Watanabe et al., 1995]; and M2 [Watanabe et al., 1995].

GPC3 mutations were previously reported in three males with SGBS [Pilia et al., 1996]. We have identified four more GPC3 mutations in nine males with clinical manifestations of SGBS. In this report, we describe the genotype/phenotype correlations of the 18 males with GPC3 deletions.

MATERIALS AND METHODS

Polymerase Chain Reaction Analysis of GPC3 Mutations

The polymerase chain reaction (PCR) primers used have been previously described [Pilia et al., 1996]. Two

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additional primer sequences were generated from the 5′ and 3′ UTR regions adjacent to exons 1 and 8 (primer A: 5′-GGTAGCTGCGAGGAAACTTTTG-3′; primer B: 5′-CCTCCTTATCGAGGAAGACCA-3′). PCR reactions were performed according to the method of Blanchard et al. [1992].

GPC3 Probes Synthesized by Reverse Transcription PCR

Total RNA was prepared from human fetal kidney tissue (18 weeks gestation) by the Trizol reagent and RNA extraction protocol (GibcoBRL, Gaithersburg, MD) and subjected to reverse transcription (RT) using the following primer pairs: A and exon 2B, exons 1A and 3B, exons 3A and 6B, as well as exons 6A and B. The resulting four overlapping cDNAs (A to exon 2, 417 bp; exons 1 to 3, 348 bp; exons 3 to 5, 982 bp; exons 5 to B, 518 bp) covered the complete 2.2 kb GPC3 transcript. The PCR products were cloned into plasmid pCRII using the TA cloning procedure (Invitrogen, San Diego, CA). The PCR products were excised from 0.6% low-melt agarose gels (GibcoBRL) and labeled with [^{32}P]dCTP using the Rediprime DNA labeling protocol (Amersham Life Sciences, Arlington Heights, IL).

Southern Blot Analysis of GPC3 Mutations

Human genomic DNA was isolated from leukocytes or fibroblasts [Sambrook et al., 1989]. DNA was digested with one of six restriction enzymes (BamH1, EcoR1, Hind III, BglII, PstI, TaqI), followed by Southern blotting and hybridization with the four RT-PCR probes. The Southern blots were washed in 0.1 × SSC with 0.1% SDS at 60°C and exposed to x-ray film for 1 to 8 days at −70°C.

RESULTS

Of the 12 families, linkage to Xq26 was established in the 2 large enough for this analysis (families A and B) (Fig. 1) [see Xuan et al., 1994]. Four males represented isolated cases of SGBS (including families C, F, and G). Three males represented isolated cases of SGBS, each with an unaffected brother who inherited the other maternal Xq26 loci (mutation not identified). Two males (families D and E) each had one affected brother who inherited the same maternal Xq26 loci. The 12th family had several affected male relatives (mutation not identified).

PCR amplification of six intra-exonic sequences identified GPC3 deletions in five unrelated SGBS families (Fig. 2). PCR results for families A, B, and C have been previously described [Pilia et al., 1996]. Southern blot analysis complemented the PCR results (families B, C, and D) and identified additional deletions in affected males from families E, F, and G (Fig. 3). No deletions were detected using intra-exonic PCR or Southern blot analysis in males from the remaining five SGBS families. In family A, exon 2 PCR primers, which generate a DNA fragment that spans approximately half the exon, detected a deletion that was not observed on Southern blot. The probe, A-2B, hybridized to DNA fragments that were greater than 7 kb in size, and we conclude that the deletion in family A is too small to result in a band shift on Southern analysis. The affected male from family G had a deletion at the 3′ end of exon 1 that was detected by Southern blot but not PCR.

To focus our search for novel mutations, the phenotypic profiles of the families were reviewed. Three males were affected in family D (Fig. 1). Although their mother (III-1) did not have manifestations of SGBS, family photographs suggested that four maternal second cousins were affected. This suggests that III-1 inherited the GPC3 mutation from her mother. In families C and E, GPC3 mutations were identified in the mothers of affected males (family C, II-1; family E, III-1) but not in the maternal grandmothers (maternal grandfathers clinically unaffected). In family C, II-1 is 1.78 m tall and has macrostomia, an enlarged mandible, and a mild eversion of the lower costal margin of the ribs. In family E, III-1 is 1.67 m tall and does not have clinical findings of SGBS. The only affected male in family F (II-3) has two unaffected brothers. His mother (I-1) is 1.57 m tall and does not have manifestations of SGBS other than a ventricular tachyarrhythmia that may be unrelated. In family G, the mother (I-1) of II-3 is 1.62 m tall and has a mildly enlarged jaw. Thus, GPC3 mutations may have arisen de novo in four of seven families. The mothers of affected males in families C and E had somatic GPC3 mutations. GPC3 mutation analysis is pending on the mothers of affected males from families F and G. Unfortunately, GPC3 is not expressed postnatally in leukocytes or fibroblasts, precluding their use for GPC3 mRNA analysis.

Clinical manifestations of the seven males with GPC3 mutations and their affected male relatives are shown in Table I. Common findings included a characteristic face that became 'coarse' during childhood, resembling mucopolysaccharide storage disorders. Enlargement of the mandible occurred later during childhood and adolescence. The site of GPC3 mutation did not predict the occurrence of cleft palate, heart defects, hernias, supernumerary nipples, and renal and skeletal abnormalities. Postnatal overgrowth was not a constant finding of SGBS during early childhood. Two males fell within the normal centile range for height and head circumference in early childhood during periods of failure to thrive secondary to poor feeding (family A, III-3) or treatment for Wilms tumour (family G, II-3). Ultimately, all males grew beyond the 95th centile in height and weight. In family A, three of six affected males demonstrated varying degrees of hypospadias, a defect not found in the other six families. Table I includes two females with SGBS phenotypes and X;autosome translocations that disrupt GPC3 [Punnett, 1994; Pilia et al., 1996]. When the clinical findings of the females were compared with those of the affected males, the GPC3 breakpoint locations did not impart distinct SGBS phenotypes. It is noteworthy that males with apparent 3′ deletions of GPC3 had phenotypes as severe as those with mutations that might be considered more disruptive (e.g., families D and F).

DISCUSSION

Recently, mutations in the GPC3 gene have been associated with SGBS [Pilia et al., 1996]. This gene spans

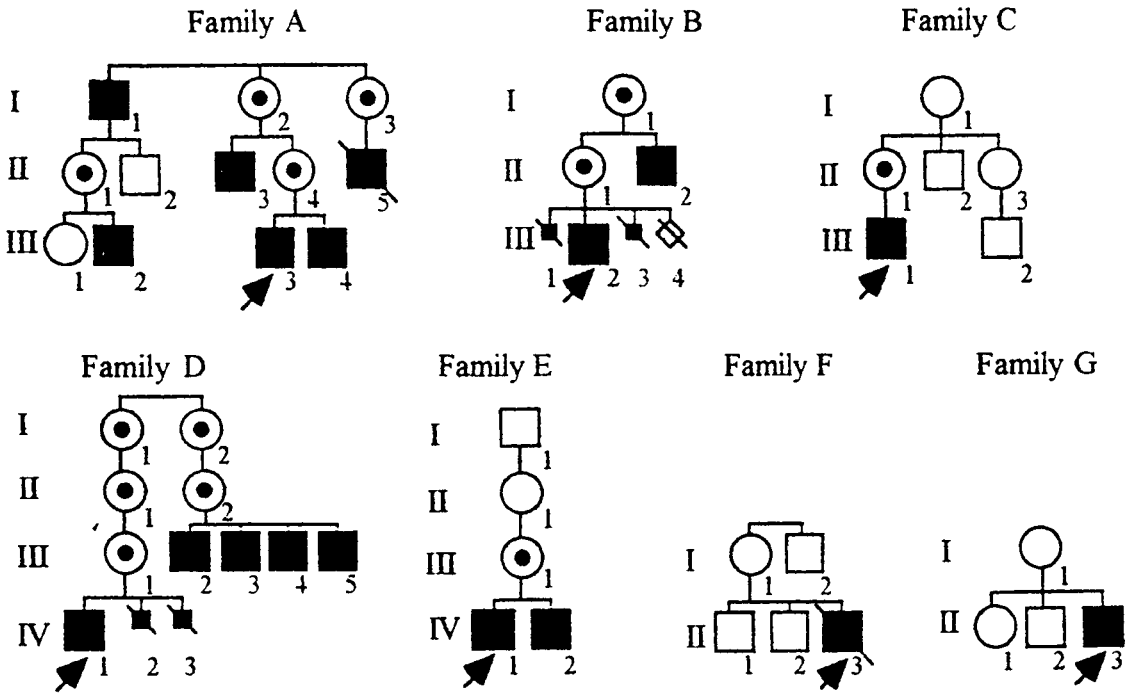

Fig. 1. Pedigrees of 7 males with SGBS and GPC3 mutations. Only the relatives discussed in this report are included. The propositi are indicated with an arrow.

more than 500 kb of genomic DNA in chromosome Xq26 and encodes a 2.2 kb cDNA. In this report, we demonstrate deletions in the GPC3 gene sequence in four additional unrelated males with clinical manifestations of SGBS (Fig. 2). Deletions were identified throughout the length of the GPC3 transcript with no evidence of mutation hot spots.

No correlation was found between the phenotype of the 18 affected males and 2 female X;autosome translocation carriers with the site or size of the GPC3 mutations. Therefore, we think that SGBS results from a loss of GPC3 function. This finding may reflect a selection bias as the individuals described in this report were chosen because they had 'typical' manifestations of SGBS. Although 'typical' SGBS may represent a complete loss of GPC3, there may exist modified SGBS phenotypes or non–SGBS-associated anomalies due to residual activity of the GPC3 gene product.

De novo GPC3 mutations were identified in families C and E (Fig. 2). Two additional families (F and G) had only one affected male and may also represent new mutations. It may be that new GPC3 mutations represent the main source of SGBS affected males. In regard to reproductive fitness, we are aware of only one affected male who has fathered children in family A (I-1). Of the 18 males described in this report, 4 died as a stillbirth or termination of pregnancy, 1 died due to sudden infant death at 4 months (family F, II-3), and 1 died of cancer (family A, II-5). Three males were successfully treated for potentially lethal conditions including congenital heart disease (family A, III-3) and cancer (family A, III-1; family G, II-3). This is similar to the 50% mortality rate reported by Neri et al. [1988] in a review of published cases of males with SGBS. For purposes of genetic counseling, SGBS may be similar to other X-linked recessive conditions such as Duchenne muscular dystrophy, in which one third of males have de novo mutations and the mothers of affected males have a two thirds chance of being heterozygous for the GPC3 mutation. Confirmation of this model will require additional mutation analysis on a larger group of SGBS families.

OCI-5, the rat homologue of GPC3, is developmentally regulated in rat and mice, with the highest levels of expression in mesenchymal tissues in the fetus [Filmus et al., 1988; Watanabe et al., 1995]. Although Northern analysis did not detect GPC3 expression in human fetal brain [Pilia et al., 1996], similar studies in mice found high mRNA levels in the embryo that were lower at postnatal day 2 and barely detectable in the adult [Watanabe et al., 1995]. In this report, SGBS males had a variety of anomalies in the tissues expected to express GPC3 during normal development, including aqueductal stenosis, abnormal lung lobation, hepatosplenomegaly, polysplenia, and nephromegaly. Renal dysplasia and cysts were observed in six males. Nephrogenic rests were described previously in SGBS and may predispose some individuals to the development of Wilms tumour [Hughes-Benzie et al., 1994]. The absence of a functional GPC3 protein may delay or alter the normal induction of nephron formation. Additional anomalies included vertebral segmentation defects, rib anomalies, smooth muscle defects in the bowel wall, and skeletal muscle hypoplasia with hypoplastic calf muscles and winged scapulae (Table I), suggesting a role for GPC3 in osteochondrogenesis and muscle formation.

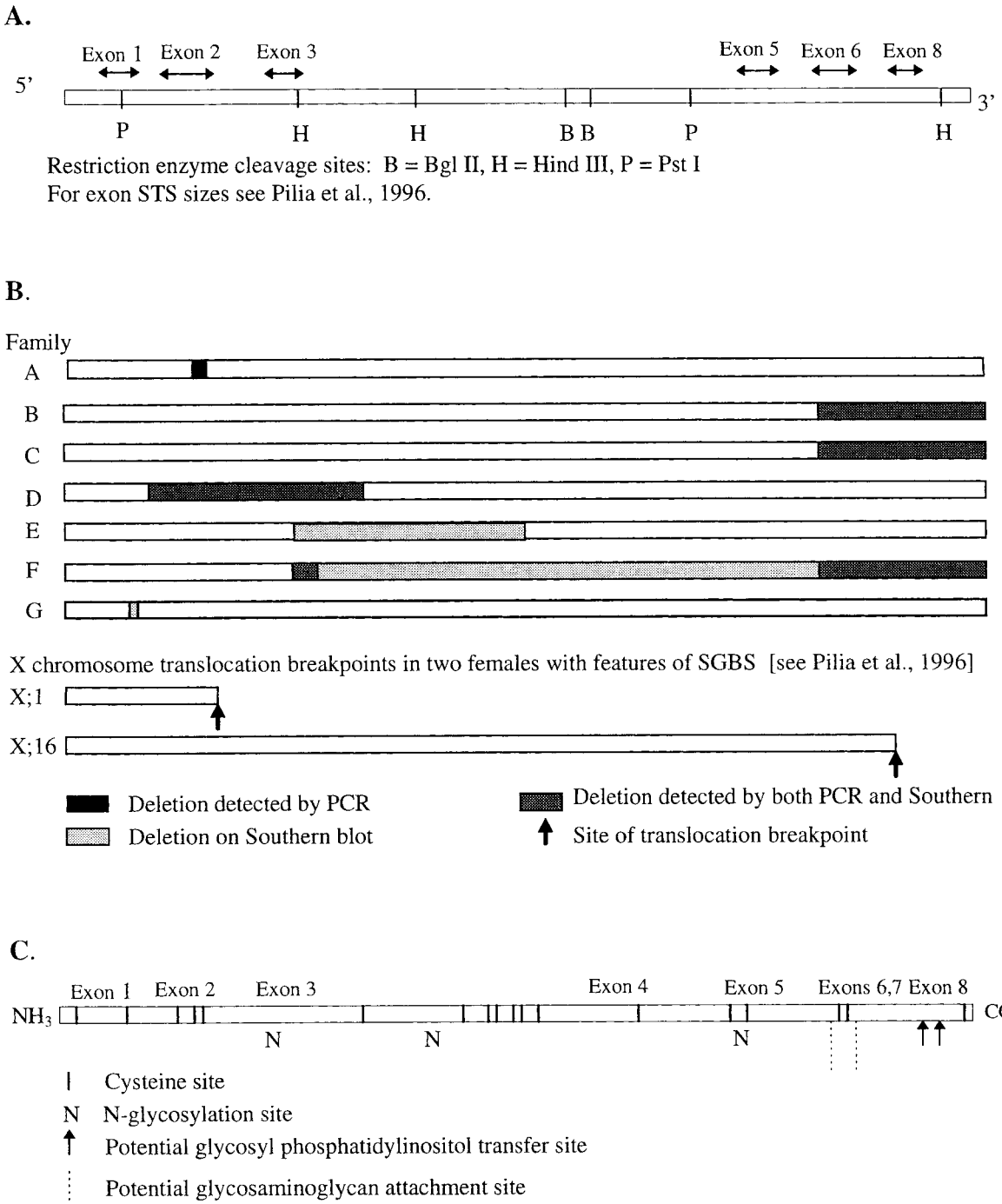

Fig. 2. cDNA and protein structure of GPC3. **A**: GPC3 cDNA (2.2 kb) showing location of intra-exonic PCR probes and restriction enzyme cleavage sites. **B**: Approximate cDNA location of GPC3 deletions in seven unrelated SGBS families. **C**: GPC3 protein (580 aa) including approximate location of exons.

GPC3 may also be involved in the induction of programmed cell death (apoptosis) in certain tissues. Apoptosis is an integral part of the normal development of the digits [Little and Mirkes, 1995]. Polydactyly and syndactyly may be the result of abnormal or delayed induction of apoptosis in the mesenchymal cells forming the interdigital and interphalangeal spaces. Similarly, supernumerary nipples may be the result of abnormal apoptosis and retention of epithelial cells in the mammary ridges.

Cleft palate is a common developmental defect seen in SGBS. Relative macroglossia can create a mechanical

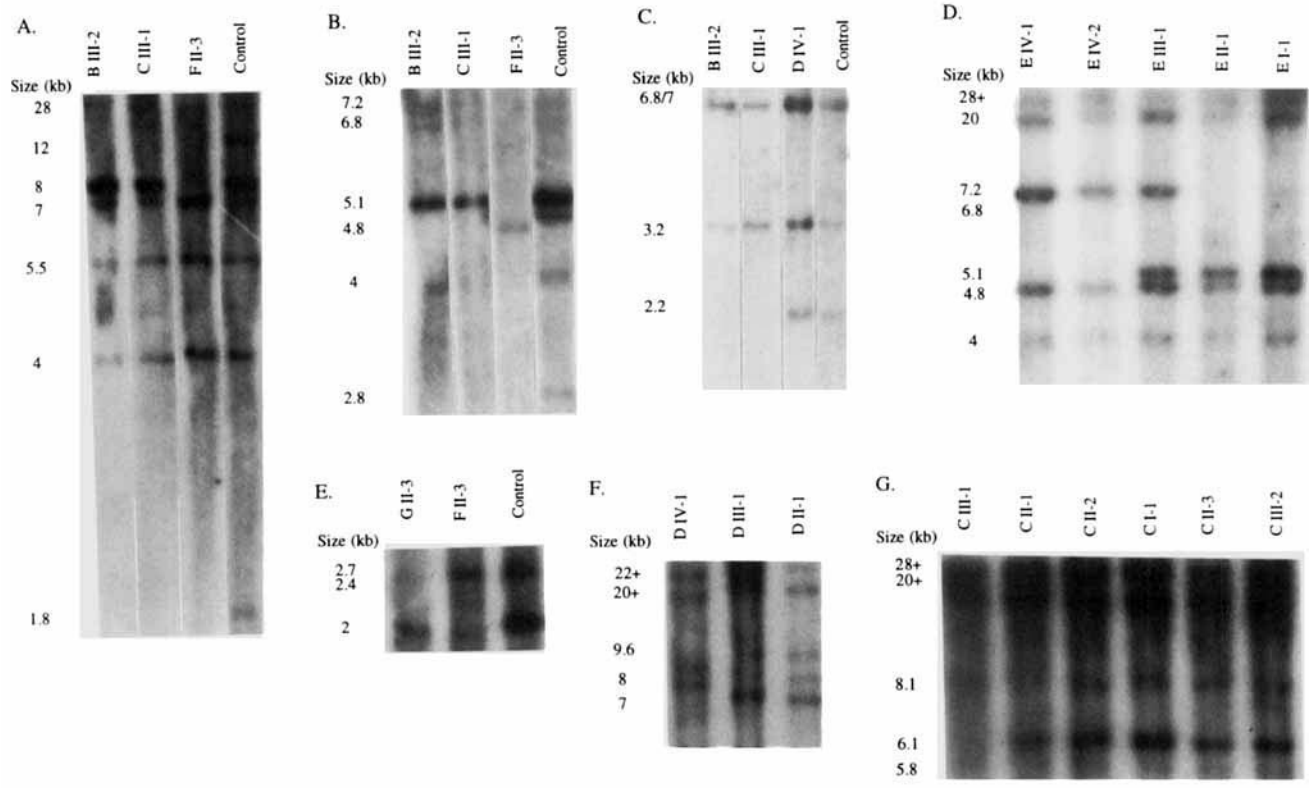

Fig. 3. Autoradiograms of Southern blots containing DNA from SGBS families probed with GPC3 cDNA. Band sizes are approximate. **A**: Bgl II digested DNA. GPC3 deletions—family B III-2 1.8, 12; family C III-1 1.8, 12; family F II-3 1.8, 8, 12, 28. **B**: PstI digested DNA. GPC3 deletions—family B III-2 2.8, 4.8; family C III-1 2.8, 4.8; family F II-3 2.8, 4, 4,8, 5.1, 6.8, 7.2, new band at 4.5. Additional bands at 0.15, 0.6, 20, 28+ not shown. **C**: HindIII digested DNA. GPC3 deletions—family B 2.2, 7; family C 2.2, 7; family D 0.9, 20+. Additional bands at 0.9, 20, 28+ not shown. **D**: PstI digested DNA from family E. GPC3 deletion—5.1, prominent 7.2 band seen in IV-1, IV-2, III-1. Additional bands at 0.15, 0.6, 2.8 not shown. **E**: TagI digested DNA. GPC3 deletions—family G II-3 2.4; family F II-3 2.4 faint, 0.1, 2.1, 2.7, 4.3, 6; Additional bands at 6.0, 4.3, 0.4, 0.3 and 0.1 not shown. **F**: EcoRi digested DNA from family D. GPC3 deletion—IV-1 7. Additional band at 4.2 not shown. **G**: BamHI digested DNA from family C. GPC3 deletion III-1 5.8, 6.1.

obstruction that prevents the normal fusion of the palatal shelves during the seventh week of gestation. However, the presence, severity, and shape of cleft palate in SGBS males did not correlate with the size of the tongue (Table I). An alternative hypothesis is that the absence of GPC3 might result in abnormal epithelial-mesenchymal interactions and a lack of appropriate cell induction and tissue fusion in the palate. In mouse embryos, antibodies to heparan sulfate-related epitopes showed the strongest staining in mesenchymal tissues of the head, limbs, lungs, gut, and kidneys that were undergoing active morphogenesis [David et al., 1992]. The most pronounced reactions were in mesenchymal cells in close proximity to epithelial tissues, especially those undergoing differentiation as a result of intense and reciprocal epithelial-mesenchymal interactions. Similarly, formation of the lip and cardiac intraventricular septum requires fusion of epithelial covered mesenchymal tissue. Abnormal cell-cell interactions in these tissues could explain the occurrence of cleft lip, lower lip groove, and cardiac defects (atrial septal defect, ventricular septal defect) in SGBS.

SGBS shares many clinical findings with Beckwith-Wiedemann syndrome (BWS) [Hughes-Benzie et al., 1992b]. Relaxation of the genetic imprinting of the insulin-like growth factor II (IGF2) gene in some cases of BWS and in isolated Wilms tumour suggests that an upregulation of IGF2 contributes to the overgrowth phenotype and increased risk of malignancy [Ogawa et al., 1993; Rainer et al., 1993; Schneid et al., 1993; Weksberg et al., 1993; Cristofori et al., 1994; Hedborg et al., 1994; Reik et al., 1994]. Recently, mice lacking the gene encoding a putatively biologically inactive mannose-6-phosphate IGF2 receptor (IGF2/Mpr) were made [Lau et al., 1994; Wang et al., 1994]. The receptor acts as a sink to remove IGF2; therefore, it is thought that these mice had a de facto IGF2 overexpression. Most affected mice died shortly after birth because of respiratory insufficiency or cardiac anomalies. Additional findings included a 25–30% increase in birth weight compared with litter mates, enlarged placentas, polydactyly, minor facial anomalies, bent tails, sternal anomalies, cardiac defects (hyperplasia of the myocardium, thinning of the intraventricular septum,

TABLE I. Clinical Manifestations of SGBS Affected Males With GPC3 Mutations and Their Affected Male Relatives*

Family and ID no.	Perinatal anomalies[c]	CNS[d]	SGBS face[e]	Palate/lip	Macroglossia	Macrognathia
A						
I-1[a]	LGA	–	+			+
II-3[a]	LGA	Speech	+	Submucous cleft palate		+
II-5	LGA	–	+	Lower lip groove		–
III-2[a]	b, g LGA	–	+	High palate	+	–
III-3[a]		Speech, gross motor	+	High palate, lower lip groove	+	–
III-4[a]	LGA	Speech	+	Cleft palate, cleft lip, lower lip groove	+/–	–
B						
II-2[a]	LGA		+	Lower lip groove		+
III-1[a]	a, TOP	v		Cleft palate		
III-2[a]	a, LGA	Speech, gross motor, aqueduct stenosis	+	Submucous cleft palate, lower lip groove	+	–
III-3	a, TOP					
C						
III-1[a]	b, g, h LGA	Speech	+	Submucous cleft palate, bifid uvula	+	–
D						
IV-1[a]	b, LGA	Speech	+	Cleft palate, bifid uvula	+	+
IV-2	SB				–	
IV-3	a, TOP, cystic hygroma, LGA			Bilateral cleft lip	–	
E						
IV-1[a]	b, h,LGA	Speech, fine motor	+	Broad palate	+	+
IV-2[a]	a, b, g, h, LGA	Gross motor[b]	+	Broad palate	+	+
F						
II-3[a]	a, b, g, h, LGA	–	+	Cleft palate, bifid uvula	+	–
G						
II-3[a]		Speech	+	High palate, lower lip groove	+	+
X;1 trans	b, LGA	Speech	+	Bifid uvula	+	+
X;16 trans	LGA		+			

*References: Family A, Hughes-Benzie et al. [1992a], Xuan et al. [1994]; Family D, Chueh et al. [1993]; Family G, Hughes-Benzie et al. [1994]; X;1 translocation female, Punnett [1994].
[a] GPC3 mutation analysis done.
[b] Due to calf muscle hypoplasia.
[c] Perinatal anomalies: a, elevated AFP; b, hyperbilirubinemia; g, hypoglycemia; h, polyhydramnios; LGA, large for gestational age; SB, stillbirth; TOP, termination of pregnancy.
[d] CNS: v, mild ventriculomegaly, developmental delay: fine motor, speech delay, gross motor delay.
[e] SGBS face: "coarse" with macrostomia.
[f] Heart anomalies: VSD, ventricular septal defect; ASD, atrial septal defect; IVC, inferior vena cava.
[g] GI: m, hypoplastic muscular layer of colon; r, rectal prolapse; hernias—d, diaphragm; i, inguinal; u, umbilical.

TABLE I. *Continued*

Heart anomalies[f]	GI[g]	GU anomalies	Extra nipples	Musculo/skeletal anomalies	Hand anomalies
–	i	Renal dysplasia, 1° hypospadias	+	Pectus excavatum, thoracic scoliosis, winged scapulae	Blunt tips, syndactyly, nail hypoplasia
–	i, r	Cryptorchidism	Areolar skin tags	Pectus excavatum, spina bifida occulta, winged scapulae	Blunt tips
–	i	Renal dysplasia, Wilms tumour	–		
–	i, u	Nephromegaly, renal dysplasia, cryptorchidism, Wilms tumour	–	Pectus excavatum, knee and elbow contractures	Blunt tips, large hands
Subaortic membrane, pulmonary stenosis, left ventricular hypertrophy, VSD, transposition of IVC/descending aorta	i, u	Hydroureters, cryptorchidism, 3° hypospadias, bifid scrotum	+	Pectus excavatum	Blunt tips, nail hypoplasia
VSD	i, u	Nephromegaly, cryptorchidism, 2° hypospadias	+	13 ribs, pectus excavatum, winged scapulae	Blunt tips, polydactyly, nail hypoplasia
Aortic stenosis		Nephromegaly, renal dysplasia		Hooked lumbar vertebrae	
VSD, ASD		Hydronephrosis			
Pulmonary artery branch stenosis	u	Nephromegaly, renal dysplasia	+	Pectus excavatum, rib anomaly	Nail hypoplasia
Murmur	i	Cryptorchidism			Polydactyly
2 vessel cords	d	–			
–	c	Nephromegaly, renal dysplasia, duplicated ureter	+	Pectus excavatum	Large hands
VSD, pulmonary stenosis	u	Nephromegaly, hydrocele	+	Prominent xiphoid, club feet, hypoplastic calf muscles	Polydactyly
Patent foramen ovale	u, m	Nephromegaly, hydronephrosis, hydroureter, nephrogenic rests	+	Pectus excavatum, bifid rib	Blunt tips, nail hypoplasia, syndactyly
Murmur	d, i	Cryptorchidism, Wilms tumour	+	–	–
Pulmonary stenosis	d, u	Nephromegaly	+	Rib anomaly	Large hands
Murmur	i	Nephromegaly		Klippel-Fiel	

tricuspid valve anomalies), and enlargement of the uterus with imperforate vagina or vulval closure. Polydactyly, sternal anomalies, and ventricular septal defects are more typical of SGBS than BWS. Ligand blotting analysis showed that GPC3 interacts with IGF2 [Pilia et al., 1996], suggesting that GPC3 binds and downregulates IGF2 in a manner postulated for IGF2/Mpr. Thus, the loss of either GPC3 or IGF2/Mpr would result in an effective increase in IGF2, something that has been suggested to occur in BWS. However, the fact that the SGBS and IGF2/Mpr null phenotypes show greater overlap with one another than with

BWS suggests that other as yet unidentified factors are involved in the pathogenic mechanisms of these disorders.

The ongoing characterization of GPC3 mutations and their correlation with the observed phenotypes (be they SGBS or possibly unanticipated clinical profiles) will aid in our understanding of somatic overgrowth and the physiologic role of this important proteoglycan.

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